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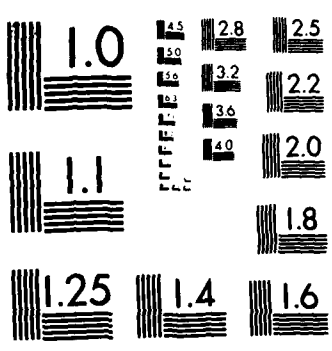

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ARO 20545.1-CS

A Basic Study to Assess the Potential Usefulness of Resonance Raman Spectroscopy as a Means of Rapidly Detecting and Identifying Bacteria and Other Microorganism.

Final Report by
Wilfred H. Nelson
Chemistry Department
University of Rhode Island
Kingston, RI 02881

February 15, 1985
U.S. Army Research Office
Research Agreement No. DAAG29-83-K-0136

A. The Problem Studied

Pathogenic bacteria constitute a well-documented serious health threat. To safeguard the public, a number of effective detection techniques have been developed, some of which are very sensitive and specific. However, commonly, no single test will allow identification of an unknown organism. Rather a complex series of tests is required which may be difficult to interpret and which often require many days or even weeks to complete.

There are many situations where public health requirements point to the need for rapid analysis of microorganisms. Recreational waters as well as drinking water can be subject to contamination which is not predictable. Water reuse especially will require reliable and rapid testing because of greater potential for contamination. A very pressing need has developed for rapid detection of airborne microorganisms as well. In addition, there are many clinical applications where rapidity and specificity of analysis is critically important, yet lacking.

Several physical methods have been applied with varying degrees of success for the "rapid" identification of microorganism. These include pyrolysis-gas chromatography mass spectroscopy (1), resonance Raman spectroscopy (2,3), fluor escence emission-excitation matrix spectroscopy (4,5), flow cytometry (6) and chemiluminescence (7). These techniques which have been reviewed recently (8), differ widely in sensitivity, speed and selectivity. This past year we have explored the potential uses of Resonance Raman and Fluoresence Lifetime Spectroscopies in the rapid characterization of bacteria.

B. Summary of Important Results

Previously we have shown (3) that chromobacteria can be distinguished on the basis of distinctive resonance Raman spectra. Spectra are excited by low power argon ion laser radiation at 488 nm and are due to the presence of carotenoid pigments. Algae have been studied by us (2) in similar fashion.

While carotenoid pigments in bacteria generally are not useful for identification purposes, the study of chromobacteria has allowed us to assess the potential sensitivy of resonance Raman spectroscopy in this application. With the aid of the research staff of Spex, Inc., Metuchen, N.J., we were able to laser illuminate and count two types of bacteria under a microscope. Because it was possible to see the laser excited bacteria while the resonance Raman spectra were being obtained, it was possible to estimate closely the number of bacteria producing a given spectrum. Spectra attached (Fig. 1) of two types of flavobacteria were the result of scattering from 25-50 organisms. Laser power was very low and moderate background fluoresence was present as interference. Thus, this experiment did not seriously press the potential detection limits.

Such high sensitivity suggests but does not prove that spectra are "surface enhanced". Preliminary studies tend not to support this explanation. Sonication of the organisms, for example, produces very little change in the intensity of the resonance Raman lines. It is clear that the resonance Raman technique can be highly sensitive and our experiments suggest that remote detection and detection from mixtures is possible in principle.

Our experiments have clearly demonstrated, however, that the future of this type of work does not lie with the use of Ar + ion lasers as the illumination source. This is so simply because most bacteria are not intensely colored

and do not give strong resonance Raman spectra in the visible range. We have considered using cytochromes as a means of detection since they do absorb in the visible . Unhappily they do not produce strong resonance Raman spectra because the electronic transitions concerned are not completely "allowed" and tend to show fairly small extinction coefficients.

Various non-fluorescent dyes have been attached to bacteria and changes in the dye Raman spectra noted. To date no dye has been found which produces a resonance Raman spectrum which is as distinctive as that of the natural carotenoids. Use of resonance Raman for bacterial analysis purposes is difficult with most "real" samples in the visible range because of the interference from fluorescence. For that reason we are not any longer pursuing the use of dyes as identifying probes in resonance Raman studies. Fluorescence due to bacterial tryptophans and flavins extends well into the "visible" range for most organisms. This produces what appears to be an unsolvable problem regarding the use of visible light excitation for resonance Raman except for chromobacteria and algae. A typical bacterial fluorescence emission spectrum is attached. The spectrum of Staphylococcus epidermidis (Fig. 2) excited at 220 nm shows a very broad, relatively featureless fluorescence between 285 nm and 600 nm. It is clear that attempts to obtain resonance Raman spectra between 285-600 nm will be difficult at best for typical organisms. The fluorescence "background" will obliterate most resonance Raman spectra since the resonance Raman spectra tend to be less intense than the fluorescence.

The spectrum of S epidermidis; on the other hand, ie., Fig. 2, is more significant in terms of what it does not show-fluorescence intensity below 275nm. Fluorescence due to tyrosine, phenylalanine and nucleic acids is very low - apparently due to quenching. Since most of the important cell nucleic

acid protein components absorb in the region below 270 nm, it is apparent that a study based on these will be very attractive. Preliminary studies (9-11) of nucleotides, tyrosine, tryptophan, DNA and living cells show that even at low power (2-10 mw) excitation at 266, 257 and 213 nm give intense characteristic spectra without significant amounts of fluoresence interference. It appears that below 250 nm there is really no fluorescence background. It is believed that resonance Raman spectra obtained in the range 200-270 will provide a wealth of information which can be used to detect and identify microorganisms.

While resonance Raman spectra obtained in the ultra-violet range should provide the basis for bacterial identification, the information contained in the omnipresent fluorescence should not be overlooked. Even though most bacteria produce very similar fluorescence emission spectra associated with their protein tryptophans and flavins they can show excitation spectra especially which are markedly different (Fig. 3). This must be due to the presence of distinctly different fluorophores in bacteria.

On closer examination, however, we noted that most organisms studied had spectra dominated by tryptophan fluorescence in the ultraviolet (300-400 nm). We have attempted to determine whether fluorescence lifetimes associated with the tryptophan fluorescence are different for different organisms (12). The intrinsic steady-state fluorescence and fluorescence decay of S. epidermis, Pseudomonas fluorescens, Enterobacter cloacae, Escherichia coli and Bacillus subtilis have been observed. Each organism exhibits a strong maximum in its emission spectrum at 330-340 nm when excited at 290 nm. Iodide quenching and denaturation experiments with 8M urea provide strong evidence for the assignment of the 330-340nm fluorescence to protein tryptophan. Most importantly the decay of this bacterial protein tryptophan fluorescence has been described

by two exporential functions in all cases. The observation that characteristic protein tryptophan fluorescence lifetimes have been obtained (Table 1) for each organism suggests that measurements of tryptophan fluorescence lifetimes may provide a basis for rapid bacterial identification. A manuscript detailing this study has been submitted for publication to Biochimica Biophysica Acta (12) and has been sent to ARO with the standard Memorandum of Transmittal as has an earlier paper dealing with this topic (13).

This fluorescense lifetime work continues with the aid of an instrument made available to us by Dr. Steven Suib of the University of Connecticut Department of Chemistry. At present, we are studying lifetimes associated with flavin fluorescence. Preliminary work shows that there are much greater ranges of lifetimes associated with flavins than with tryptophan (e.g. 0.5-17ns) Fig 4-5. Thus, there is much reason to be optimistic about the use of fluorescence lifetime studies for the identification of bacteria.

Finally, it should be noted that both resonance Raman and time-resolved fluorescence spectra will be most effectively excited by a pulsed source in the sub-nanosecond time domain. The availability of a reliable, tunable source would make possible the simultaneous determination of both types of spectra with separate detectors. Thus, a single source and instrument potentially could assess the identity, number and viability of microorganisms.

C. List of ALL PUBLICATIONS AND REPORTS.

1. The Rapid Identification of Bacteria Using Time-Resolved Fluorescence and Fluorescence Excitation Spectral Methods. S. K. Brahma, M. P. Baek, D. Gaskill, R. K. Force, W. H. Nelson and J. Sperry. Applied Spectroscopy (1985)

2. The Potential Rapid Identification of Bacteria Through Measurement of Protein Tryptophan Fluorescence Decay Lifetimes. R. A. Dalterio, W. H. Nelson, D. Britt, J. Sperry, D. Psaras, J. F. Tanguay and S. L. Suib. Biochimica Biophysica Acta (1985)

3. The Detection and Identification of Bacteria by Resonance Raman and Time-Resolved Fluorescence Spectroscopies, W. H. Nelson. Abstracts 3rd Biodetection Conference sponsored by A.R.O., Raleigh, N.C., April 1984.

D. Participating Scientific Personnel.

1. W. H. Nelson*, Principal Investigator, URI, Chemistry
2. J. Sperry, Co-investigator, URI, Microbiology
3. S. K. Brahma*, Postdoctoral, URI, Chemistry
4. R. A. Dalterio*, Postdoctoral, URI, Chemistry
5. D. Britt*, Graduate Student, URI, Microbiology
6. D. Gaskill, Undergraduate Student, URI, Chemistry
7. M. P. Baek*, Graduate Student, URI, Chemistry
8. D. Psaras, Graduate Student, U. Conn., Chemistry
9. J. F. Tanguay, Graduate Student, U. Conn., Chemistry
10. R. K. Force, Assoc. Professor, URI, Chemistry
11. S. L. Suib, Assoc. Professor, U. Conn., Chemistry

*Employed on the project
None of the participants earned a degree this past year except D. Gaskill who earned a B.Sc.

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Table I Tryptophan and Bacterial Protein Trp Fluorescence Decay Times (ns) in pH 6.6 Phosphate Buffer with NaCl, and with KI[a]

Sample	pH 6.6 Buffer only			Buffer and 0.2M NaCl			Buffer and 0.2MKI			Relative change in τ_1 with Quenching Divided by Relative change in in τ_2[c].
	τ_1	τ_2	%F(τ_1)[b]	τ_1	τ_2	%F(τ_1)[b]	τ_1	τ_2	%F(τ_1)[b]	
Tryptophan	0.79	3.06	12.3				1.00	-	100	
S. epidermidis	2.22	5.08	58.7	2.14	4.97	62.6	1.08	3.80	53.3	2.10
P. fluorescens	1.93	5.35	46.0	2.32	5.52	48.0	1.84	4.68	53.2	1.36
E. cloacae	2.24	6.02	52.2	2.32	6.07	57.8	1.93	4.68	57.0	0.73
E. coli	2.01	5.41	46.8	2.15	5.42	51.2	1.88	4.70	50.0	0.94
B. subtilis	2.27	6.01	49.7	1.90	5.39	48.8	1.72	4.66	57.0	0.70

[a]Average of two determinations made on the entire set of bacterial samples on two separate days and 1 month apart. The bacteria cultures were grown the day before the measurements were made. Reproducibility of τ for duplicate measurements on samples prepared from different batches of bacteria was ±0.09ns, average deviation from the mean, for buffer-only and buffer-NaCl suspensions. For suspensions with KI, the reproducibility, was ±0.23ns. Reproducibility was ±5% for %F(τ_1).

[b]%F(τ_1) = the percent of fluorescence intensity from the component with τ_1. %F(τ_1) + %F(τ_2) = 100

[c]This value is calculated by: $\tau_1-\tau_1(Q)/\tau_1$ / $\tau_2-\tau_2(Q)/\tau_2$.

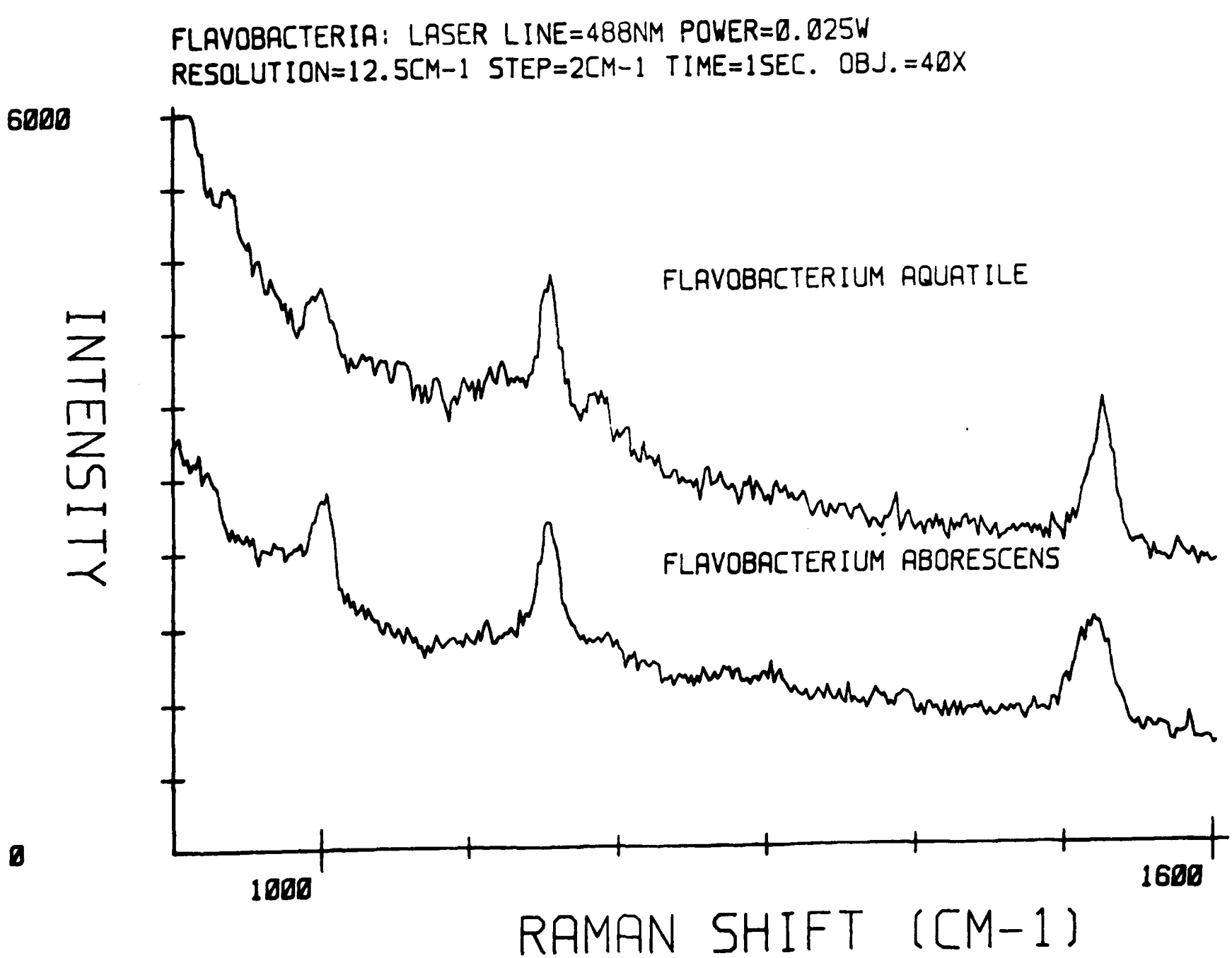


Figure 1

Figure

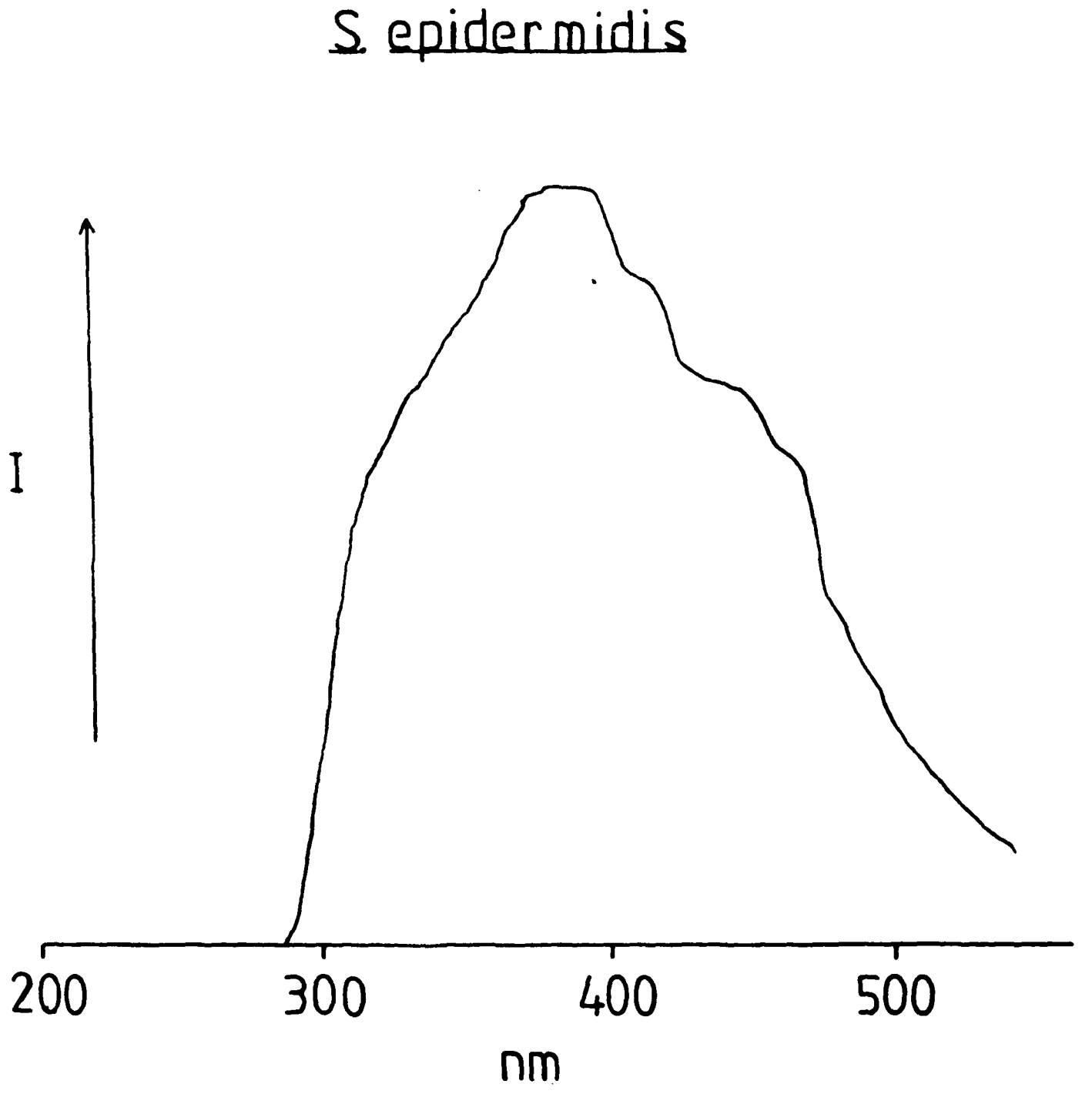


Figure 2

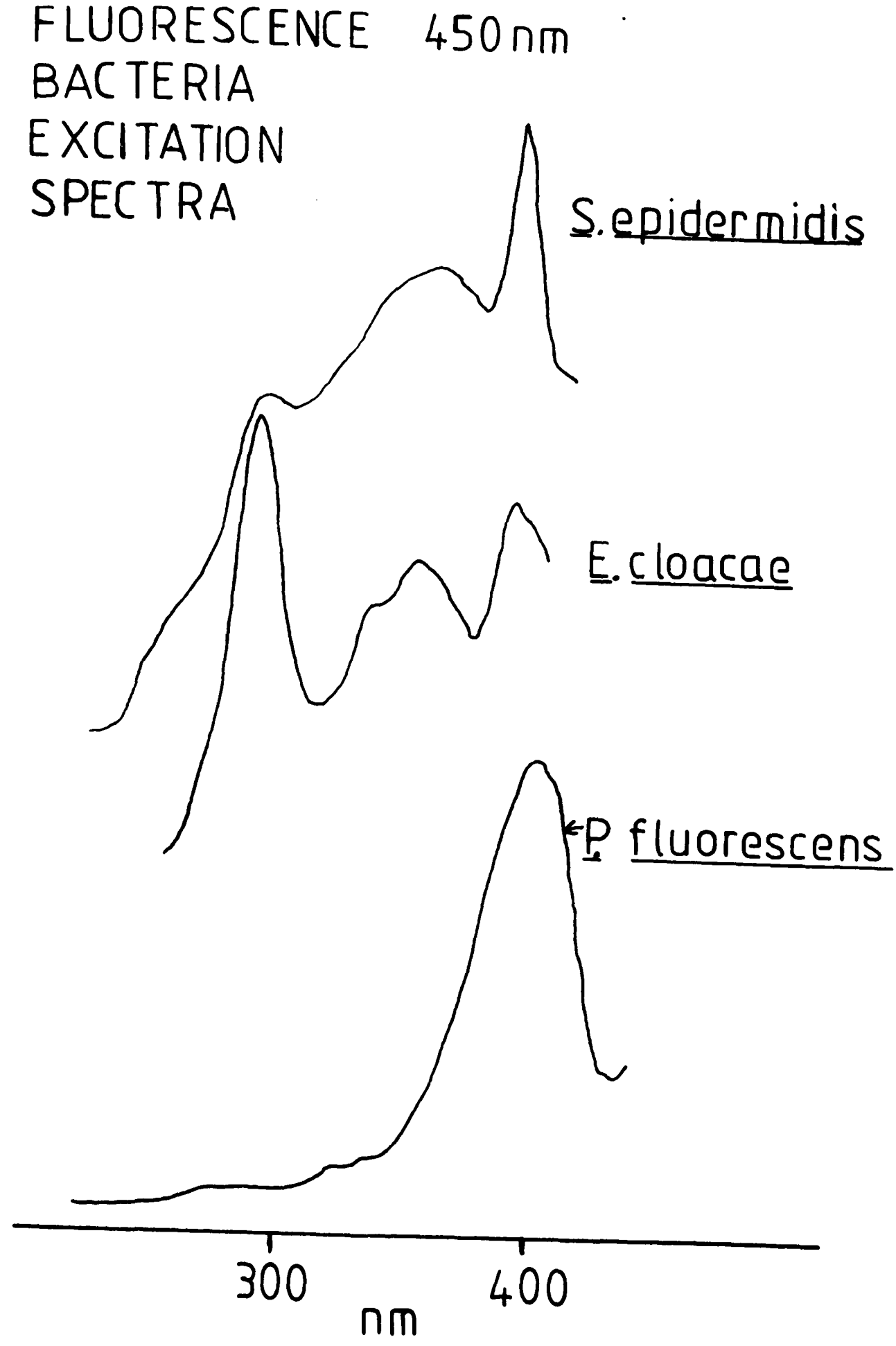
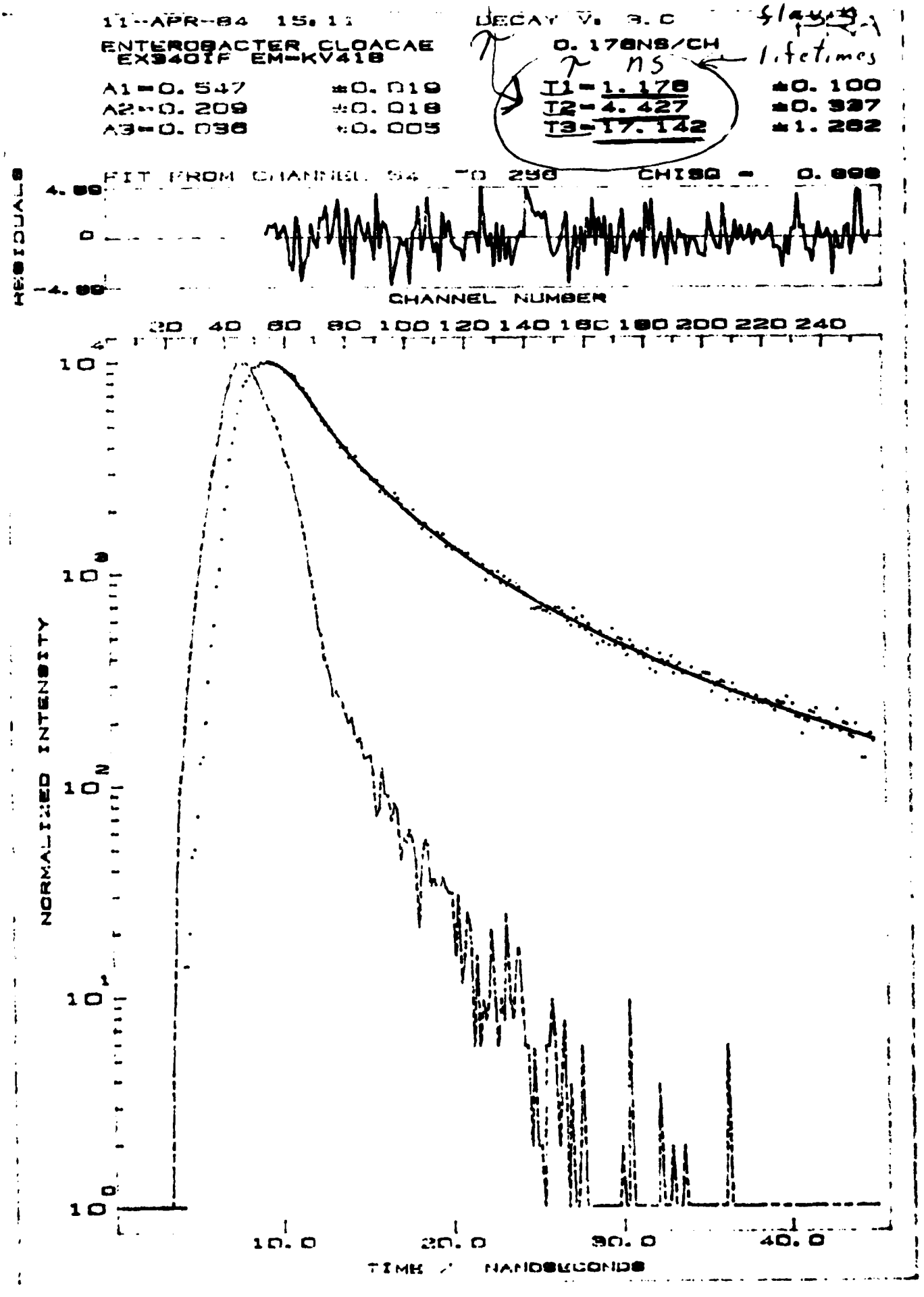

FLUORESCENCE 450nm
BACTERIA
EXCITATION
SPECTRA
S. epidermidis
E. cloacae
P. fluorescens
300
400
nm

Figure 3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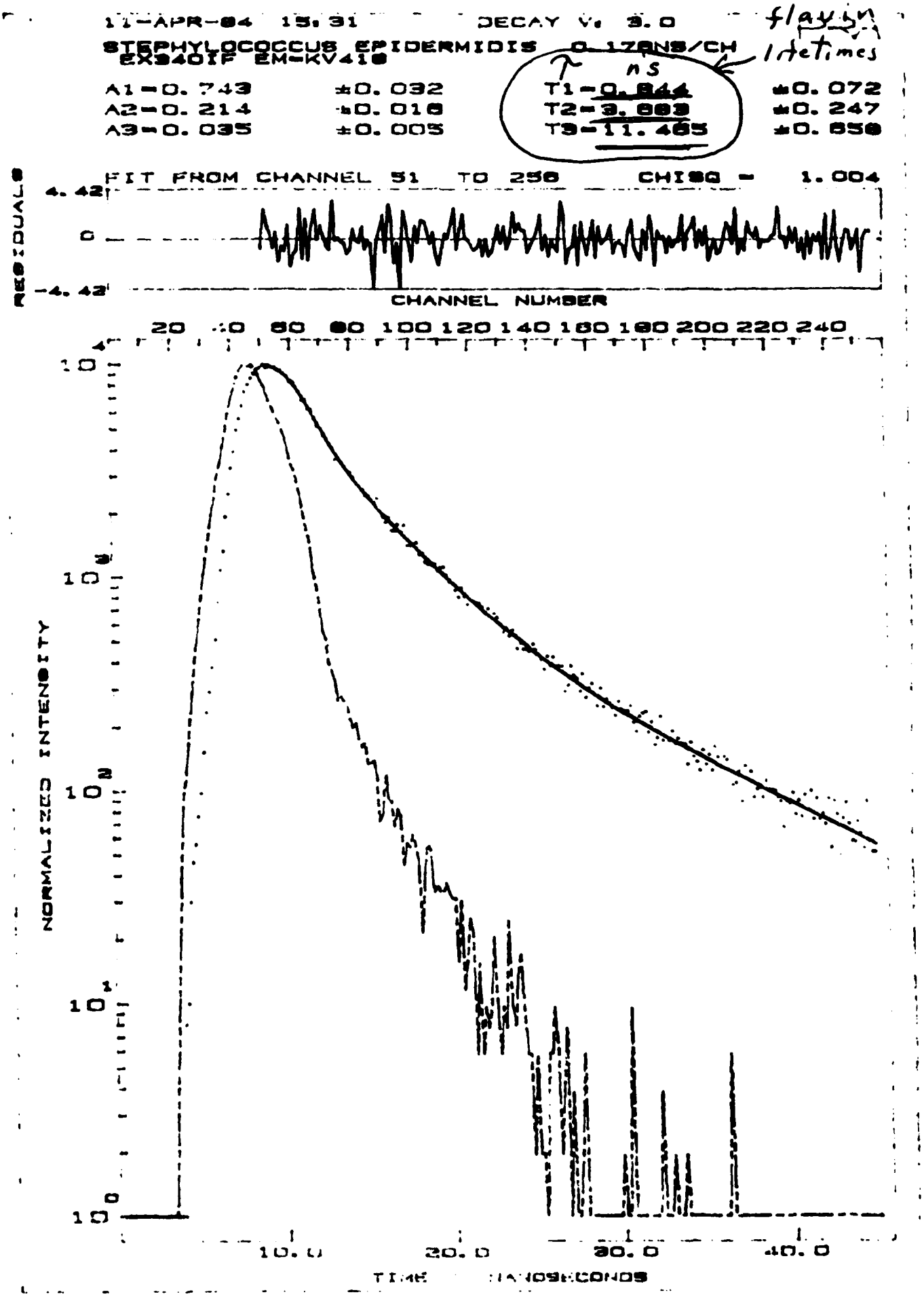
DECAY V: 3.C
ENTEROBACTER CLOACAE
EX340IF EM-KV418
0.178NS/CH
A1=0.547 ±0.019
A2=0.209 ±0.018
A3=0.036 ±0.005
T1=1.178 ±0.100
T2=4.427 ±0.327
T3=17.142 ±1.262
FIT FROM CHANNEL 34 TO 256 CHISQ = 0.000
RESIDUALS
4.00
0
-4.00
CHANNEL NUMBER
20 40 60 80 100 120 140 160 180 200 220 240
NORMALIZED INTENSITY
10.0 20.0 30.0 40.0
TIME / NANOSECONDS

Figure 4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DECAY V: 3.0
flavin
STEPHYLOCOCCUS EPIDERMIDIS 0.1ZNS/CH
4 lifetimes
EX340IF EM-KV418
ns
A1=0.743 ±0.032
A2=0.214 ±0.018
A3=0.035 ±0.005
T1=0.844 ±0.072
T2=3.883 ±0.247
T3=11.465 ±0.858
FIT FROM CHANNEL 51 TO 256 CHISQ = 1.004
RESIDUALS
4.42
0
-4.42
CHANNEL NUMBER
20 40 60 80 100 120 140 160 180 200 220 240
NORMALIZED INTENSITY
10.0
20.0
30.0
40.0
TIME NANOSECONDS

Figure 5

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